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Cellular Expression of Alphaherpesvirus gD Interferes with Entry of Homologous and Heterologous Alphaherpesviruses by Blocking Access to a Shared gD Receptor

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Several human and animal alphaherpesviruses can enter cells via human herpesvirus entry mediator C (HveC), a receptor for viral glycoprotein D (gD). In previous studies with cells expressing unknown entry mediators, cellular expression of alphaherpesvirus gD was shown to inhibit entry of the homologous virus and sometimes also of heterologous alphaherpesviruses. To investigate the mechanism of gD-mediated interference and the basis for cross-interference among alphaherpesviruses, HveC was expressed in cells as the sole entry mediator, in the presence or absence of one of the gDs encoded by herpes simplex virus type 1, pseudorabies virus, or bovine herpesvirus type 1. Cells expressing HveC alone were highly susceptible to entry of all three viruses, whereas cells coexpressing HveC and any one of the gDs were at least partially resistant to infection by each virus. Coexpression of gD with HveC did not cause reduced levels of cell-surface HveC but the HveC had reduced ability to bind to exogenous gD. Coimmunoprecipitation experiments revealed that HveC was complexed with gD in lysates of cells expressing both. Thus, cellular expression of gD can interfere with alphaherpesvirus entry by blocking ligand-binding sites of the gD receptor(s) used for entry and cross-interference can occur because different forms of alphaherpesvirus gD can compete for shared entry receptors.

INTRODUCTION

Expression of the virion envelope glycoprotein D (gD) is limited to the alphaherpesvirus subfamily of the herpesvirus family. All alphaherpesviruses studied to date, except varicella-zoster virus, express some form of gD. New findings with herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) demonstrate that viral gD binds to specific cell-surface receptors to determine which cell types the viruses can enter (Cocchi *et al.*, 1998; Geraghty *et al.*, 1998; Krummenacher *et al.*, 1998; Montgomery *et al.*, 1996; Warner *et al.*, 1998; Whitbeck *et al.*, 1997). Thus, this glycoprotein is an important determinant of the biology of these viruses, which typically cause lesions on mucosal surfaces and spread to the peripheral nervous system to establish latent infections in neurons but can also cause severe central nervous system disease. Because animal alphaherpesviruses such as pseudorabies virus (PRV) and bovine herpesvirus type 1 (BHV-1) can use a subset of the human gD receptors for entry into cells (Cocchi *et al.*, 1998; Geraghty *et al.*, 1998; Warner *et al.*, 1998), it seems likely that the gDs encoded by these viruses also bind to the receptors and to homologous porcine and bovine receptors.

There are similarities among these alphaherpesviruses in viral and cellular requirements for entry (Mettenleiter, 1995; Spear, 1993). Binding of these viruses to cells is usually mediated by interactions of viral gC, and perhaps also gB, with heparan sulfate chains on cell-surface proteoglycans (Herold *et al.*, 1991, 1994; Mettenleiter *et al.*, 1990; Okazaki *et al.*, 1991). Then, gD can bind to any one of several specific cell-surface receptors (Cocchi *et al.*, 1998; Geraghty *et al.*, 1998; Krummenacher *et al.*, 1998; Montgomery *et al.*, 1996; Warner *et al.*, 1998; Whitbeck *et al.*, 1997). The gD–receptor interaction triggers penetration of virus, which occurs by fusion of the viral envelope with a cell membrane and requires the concerted action of four viral glycoproteins, gB, gD, gH, and gL (for reviews see Mettenleiter, 1995; Spear, 1993; Tikoo *et al.*, 1995). Thus, efficient entry of the gD-expressing alphaherpesviruses into cells depends on the cell-surface expression of (i) heparan sulfate, a carbohydrate component of ubiquitous cell-surface proteoglycans, and (ii) gD-binding receptors, which probably have a more limited distribution.

Five distinct gD receptors capable of mediating entry of HSV-1, HSV-2, PRV, or BHV-1 have been identified to date. They include four human cell-surface glycoproteins, designated herpesvirus entry proteins A–D (HveA, HveB, HveC, and CD155-HveD). HveA (officially named TNFRSF14) is a member of the tumor necrosis factor receptor family and mediates the entry of most strains of HSV-1 and HSV-2 except for HSV-1 strains with certain mutations in gD (amino acid substitutions at position 27, referred to here as Rid mutations) (Montgomery *et al.*,

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1996). HveB and HveC [also known as poliovirus receptor-related proteins 2 and 1 (Eberlé *et al.*, 1995; Lopez *et al.*, 1995) or nectin 2 and 1 (Takahashi *et al.*, 1999), respectively] are members of the immunoglobulin superfamily and, along with the poliovirus receptor (Mendelsohn *et al.*, 1989) (designated CD155-HveD), form a group of related proteins that mediate alphaherpesvirus entry (Cocchi *et al.*, 1998; Geraghty *et al.*, 1998; Warner *et al.*, 1998). Expression of HveB in normally resistant cells renders these cells susceptible to entry of HSV-1 strains with Rid mutations in gD, as well as HSV-2 and PRV (Warner *et al.*, 1998). HveC mediates the entry of all strains of HSV-1, HSV-2, PRV, and BHV-1 tested (Geraghty *et al.*, 1998). An alternatively spliced isoform of HveC has also been shown to mediate entry of HSV-1, HSV-2, and BHV-1 (Cocchi *et al.*, 1998). CD155-HveD mediates the entry of PRV and BHV-1 but has no activity for HSV-1 or HSV-2 (Geraghty *et al.*, 1998). Evidence that these cell-surface proteins are gD receptors comes from findings that (i) alterations in gD can influence receptor usage (Montgomery *et al.*, 1996; Warner *et al.*, 1998), (ii) purified soluble forms of HveA and HveC bind directly to recombinant HSV-1 or HSV-2 gD *in vitro* (Krummenacher *et al.*, 1998; Whitbeck *et al.*, 1997), and (iii) antibodies specific for HSV gD but not other envelope glycoproteins can block the binding of soluble receptors to HSV-1 virions (Krummenacher *et al.*, 1998; Nicola *et al.*, 1998). Mouse forms of HveA, HveB, and HveC have also been shown to mediate entry of one or more of the alphaherpesviruses mentioned above, with slight differences in specificity (Shukla *et al.*, 1999a; Shukla and Spear, manuscript in preparation). Nonhuman primate cells probably also express receptors that can mediate the entry of more than one alphaherpesvirus. HSV-1 and PRV were shown to compete for sites necessary for entry into Vero cells, provided the virions contained gD (Lee and Fuller, 1993).

The fifth gD receptor is a specific site or set of sites generated in heparan sulfate by the action of specific glucosaminyl 3-*O*-sulfotransferases (Shukla *et al.*, 1999b). In the absence of all the entry mediators described above (HveA-D), resistant cells can be made susceptible to HSV-1 entry, but not entry of HSV-2, PRV, or BHV-1, by expression of one of these sulfotransferases, which modify heparan sulfate so that gD can bind.

Expression of HSV-1, HSV-2, PRV, or BHV-1 gD in normally susceptible cells can render the cells resistant to infection by the homologous alphaherpesvirus and heterologous alphaherpesviruses, although reciprocal cross-interference was not always observed (Campadelli-Fiume *et al.*, 1988; Chase *et al.*, 1989, 1990, 1993; Dasika and Letchworth, 1999; Dean *et al.*, 1995; Johnson and Spear, 1989; Petrovskis *et al.*, 1988; Tikoo *et al.*, 1990). This gD-mediated interference was postulated to occur through a gD-receptor interaction that sequesters receptor within the cell, such that it is inaccessible to added virus, in a manner analogous to envelope glycoprotein-mediated interference observed in retroviruses (Delwart and Panganiban, 1989; Jabbar and Nayak, 1990; Kawamura *et al.*, 1989; Stevenson *et al.*, 1988). An alternative mechanism proposed for gD-mediated interference is an interaction between cell-associated gD and gD in virions that blocks entry (Campadelli-Fiume *et al.*, 1990). In the cited studies gD-mediated interference was investigated in cells that express unidentified gD receptors and possibly multiple entry receptors.

Aims of this study were to determine whether cross-interference among alphaherpesviruses could be observed when a single shared entry receptor, HveC, was coexpressed with different alphaherpesvirus gDs and whether gD-mediated interference could be explained by interactions of cell-associated gD with HveC. We show here that each alphaherpesvirus gD tested interfered with HveC-mediated entry of both homologous and heterologous viruses. The degree of interference depended on the source of gD. In every case HveC was expressed on the surfaces of cells coexpressing gD but the HveC had reduced receptor activity as evidenced by reduced ability to bind exogenous gD. Coimmunoprecipitation experiments demonstrated an interaction between HveC and HSV-1 gD. We conclude that gD-mediated interference can result from cellular gD–receptor interactions that block binding of receptor to virion gD. Cross-interference can be accounted for by ability of different alphaherpesviruses to use a shared gD receptor, such as HveC, for entry and to express forms of gD that compete for access to the receptor.

RESULTS

Cellular expression of alphaherpesvirus gD interferes with HveC-mediated entry of homologous and heterologous alphaherpesviruses

Chinese hamster ovary (CHO) cells are resistant to the entry of HSV-1, Rid mutants of HSV-1, PRV, and BHV-1, but become susceptible to entry of all these viruses when HveC is expressed. This makes it possible to test the ability of various members of the alphaherpesvirus gD family to interfere with viral entry via a single defined receptor, by comparing the susceptibility of cells expressing HveC alone and cells expressing both HveC and gD to entry of several alphaherpesviruses. The CHO-K1 cells were transfected with an HveC-expressing plasmid mixed in a 1:4 molar ratio with one of the gD-expressing plasmids listed in Table 1 or with control plasmid. The transfected cells were then inoculated with serial dilutions of HSV-1(KOS), HSV-1(KOS)Rid1, PRV(Kaplan), and BHV-1(Cooper) recombinants that express β-galactosidase upon entry into cells. As shown in Fig. 1, cells expressing HveC and any of the alphaherpesvirus gDs were less susceptible to infection than cells expressing HveC without gD. The various forms of gD displayed reproducible differences in ability to inter-

TABLE 1

Plasmids Expressing Entry Receptors or Alphaherpesvirus gD[a]

Plasmid[b]	Protein expressed
MW20	HveB
BG38	HveC
CJ4	HveC-HA
BG58	BHV-1 gD
CJ1	HSV-1(KOS)Rid1 gD
CJ3	HSV-1(Patton) gD[c]
CMV gD	PRV gD
pcDNA3	—

[a] Construction of plasmid or reference for plasmid under Materials and Methods.

[b] All plasmids contain the human cytomegalovirus immediate early promoter for efficient expression in eukaryotic cells.

[c] Referred to as wild-type HSV-1 gD in the text. The Patton and KOS forms of gD differ by one amino acid in the cleaved signal sequence and therefore the mature form of HSV-1(Patton) gD is identical to HSV-1(KOS) gD.

fere with HveC-mediated entry. Cells expressing HveC and HSV-1(KOS)Rid1 gD were invariably the least susceptible to alphaherpesvirus entry (maximal interference), regardless of the challenge virus, while cells expressing HveC and BHV-1 gD were the most susceptible to alphaherpesvirus entry (minimal interference).

The inhibitory effect of gD expression on HveC-mediated viral entry was dependent on the amount of gD-expressing plasmid used and presumably on the level of gD expression in relation to HveC expression, i.e., more inhibition was observed at a 1:4 ratio of HveC- to gD-expressing plasmids than at a 1:1 ratio when the total amounts of plasmid DNA and HveC-expressing plasmid were kept constant (data not shown). Expression of gD with or without HveC had no inhibitory effect on the ability of viruses in general to enter CHO-K1 cells. For example, cells transfected with mixtures of plasmids as described in Fig. 1 or with gD-expressing plasmids alone were equivalently susceptible to entry of a Sindbis virus recombinant that expresses green fluorescent protein (flow cytometry revealed that about 30% of cells were fluorescent in all cases, data not shown). Finally, the inhibitory effect of gD depended on the entry receptor expressed and whether the cell-associated gD would be expected to interact with the entry receptor. HveB mediates entry of Rid mutants of HSV-1(KOS) but not the wild-type virus. When CHO-K1 cells were cotransfected with a plasmid expressing HveB and plasmids expressing mutant or wild-type gD or control plasmid, entry of HSV-1(KOS)Rid1 was inhibited in cells expressing HSV-1(KOS)Rid1 gD but not in cells expressing wild-type HSV-1 gD (Fig. 2). Because wild-type HSV-1 gD interferes with HveC-mediated but not HveB-mediated entry, it seems likely that the effects observed in Fig. 1 are related to specific gD-receptor interactions and are not due to any nonspecific toxic effects of gD.

Expression of HveC and gD in cotransfected cells and on cell surfaces

It is generally accepted that, in cells transfected with mixtures of plasmids, the cells will take up and express either all plasmids or none. To determine whether this was true under the experimental conditions used here, two experiments were done. First, CHO-K1 cells were cotransfected with plasmids expressing HveC and HSV-1 gD at a 1:4 molar ratio, under exactly the same conditions used for the interference experiments, and analyzed by two-color immunofluorescence. Both before and after fixation, samples of the transfected cells were stained with polyclonal anti-HveC (chicken) and anti-gD (rabbit) antibodies using fluorescein-labeled and rhodamine-labeled secondary antibodies, respectively. Figure 3A demonstrates that about 50% of the transfected cells expressed proteins encoded by the plasmids on their surfaces and that cells expressing HveC coexpressed gD and vice versa. Examination of fixed cells suggested that HveC and gD were largely colocalized inside the cells. An example is shown in Fig. 3B. Second,

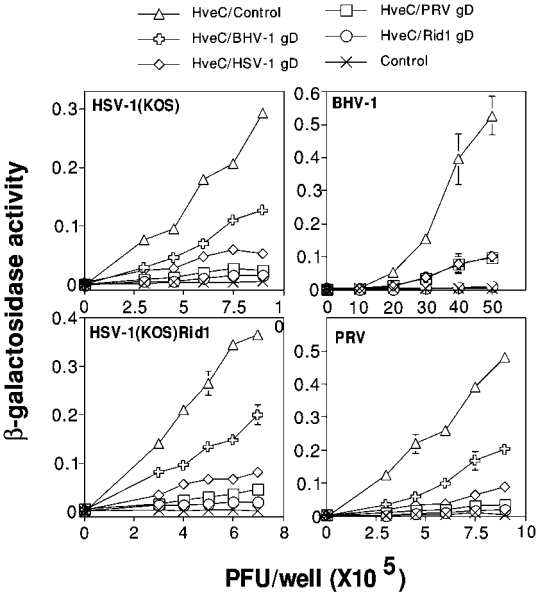

FIG. 1. Expression of alphaherpesvirus gD interferes with alphaherpesvirus entry via HveC. CHO-K1 cells were transfected with plasmids expressing HveC (pBG38) and an alphaherpesvirus gD (see Table 1) at a molar ratio of 1:4, HveC (pBG38) and control plasmid DNA (pcDNA3) at a molar ratio of 1:4, or control plasmid (pcDNA3). Twenty-four hours later the cells were replated in 96-well plates and the next day exposed to HSV-1(KOS), HSV-1(KOS)Rid1, PRV(Kaplan), or BHV-1(Cooper) recombinants expressing β-galactosidase. Six hours after inoculation, cells were lysed and β-galactosidase activity was determined as a measure of virus entry. The assays were performed in triplicate and repeated three times with similar results. The mean values plus standard deviations for a representative experiment are depicted. Transfections with alphaherpesvirus gD plasmids (see Table 1) plus control plasmid (pcDNA3) at a molar ratio of 4:1 yielded results equivalent to transfection with control plasmid (pcDNA3) alone and are not shown. PFU, plaque-forming units.

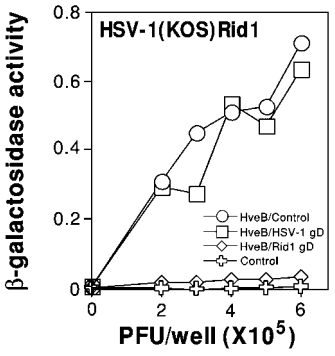

FIG. 2. Interference depends on specific gD-receptor interaction. CHO-K1 cells were transfected with plasmids expressing HveB (pMW20) and wild-type or Rid1 HSV gD (pCJ3 or pCJ1) at a molar ratio of 1:4, HveB (pMW20) and control plasmid (pcDNA3) at a molar ratio of 1:4, control plasmid (pcDNA3) and gD-expressing plasmid (pCJ3 or pCJ1) at a molar ratio of 1:4, or control plasmid (pcDNA3). The cells were inoculated with an HSV-1(KOS)Rid1 recombinant expressing β-galactosidase, HSV-1(KOS)Rid1tk12. The infections were performed in triplicate and repeated three times with similar results. The mean values plus standard deviations for a representative experiment are depicted. Transfections with wild-type HSV-1 or HSV-1(KOS)Rid1 gD plasmids, pCJ3 or pCJ1, plus control plasmid (pcDNA3) at a molar ratio of 4:1 yielded results equivalent to transfection with control plasmid (pcDNA3) alone and are not shown.

flow cytometry was performed to obtain more quantitative assessments. Dead cells were identified by propidium iodide staining and excluded from the analysis of live cells stained with anti-HveC and anti-gD antibodies. Four cell populations, transfected with control plasmid alone, HveC-expressing plasmid plus control plasmid, a plasmid expressing HSV-1(KOS)Rid1 gD plus control plasmid, or HveC-expressing plasmid plus the Rid1 gD-expressing plasmid, were analyzed. As expected, cells stained with anti-HveC but not anti-gD antibodies were detected in the population transfected with the HveC-expressing plasmid plus control plasmid and cells stained with anti-gD but not anti-HveC antibodies were detected in the population transfected with the gD-expressing plasmid plus control plasmid. Few if any singly labeled cells were detected in the population transfected with both the HveC- and the gD-expressing plasmids. The percentages of cells stained with anti-HveC antibodies were similar, about 15–20%, whether or not gD was coexpressed. These results indicate that cotransfected cells expressed both proteins on cell surfaces and that coexpression did not abrogate cell-surface expression of either.

To determine the levels of HveC and alphaherpesvirus gD expressed on the surfaces of cotransfected cells, an ELISA assay on live cells (CELISA) (Walker *et al.*, 1992) was performed in parallel with the infectivity assays described in Fig. 1. In the CELISA assay, transfected cells were incubated with either polyclonal chicken antibodies against HveC or an anti-gD antibody before fixation to ensure detection of cell-surface protein. Binding of the primary antibodies was detected after fixation by applying biotinylated secondary antibodies, streptavidin-conjugated horseradish peroxidase, and substrate. Figure 4A shows that cells expressing HveC and alphaherpesvirus gD displayed approximately the same levels of HveC as cells expressing HveC alone. The levels of cell-surface HveC expression were apparently unaffected even on cells coexpressing HveC and HSV-1(KOS)Rid1 gD, despite the virtual elimination of alphaherpesvirus entry activity observed in replicate cultures of cells (Fig. 1). Also, cells expressing a particular alphaherpesvirus gD displayed equivalent levels of cell-surface gD in the presence or absence of HveC expression (Fig. 4B).

A control experiment revealed that the CELISA assay for detection of HveC gave signals proportional to the actual level of HveC expression on cell surfaces. CHO-K1 cells were cotransfected with HveC-expressing plasmid and control plasmid (to mimic the conditions used in our interference experiments) or were transfected with control plasmid alone. The two cell populations were then mixed in variable proportions, keeping the total cell number constant, and replated in replicate 96-well dishes. One set of cultures was used for an anti-HveC CELISA assay and the other for challenge with HSV-1(KOS)Rid1 virus to quantitate viral entry activity. The results shown in Fig. 5 demonstrate that expression of cell-surface HveC and viral entry activity in the mixed-cell populations were both roughly proportional to the numbers of HveC-expressing cells added. Therefore, if the resistance to viral entry of cells coexpressing HveC and gD (Fig. 1) were due to significant reductions in amounts of HveC on cell surfaces, we would have detected these reductions in the experiment shown in Fig. 4.

Receptor activity of cell-surface HveC in the presence or absence of coexpressed gD

Because of the reduced ability of alphaherpesviruses to enter cells coexpressing gD and HveC, despite detectable cell-surface expression of HveC, an assay was devised to assess the gD-binding activity of cell-surface HveC. This gD-binding assay was performed on replicate cultures of transfected cells prepared at the same time as those used for the experiments shown in Figs. 1 and 4. A gD:Fc hybrid protein containing the extracellular domain of HSV-1(KOS) gD fused to the rabbit IgG Fc region was used as a source of soluble gD to test the binding activity of cell-surface HveC in the presence or absence of gD coexpression. The purified gD:Fc hybrid protein was shown by ELISA to contain domains recognized by gD monoclonal antibodies as well as rabbit-Fc-specific antibodies (A. Fridberg, R. J. Geraghty, B. Lum, and P. G. Spear, manuscript in preparation). Transfected cells were incubated with the gD:Fc hybrid protein and

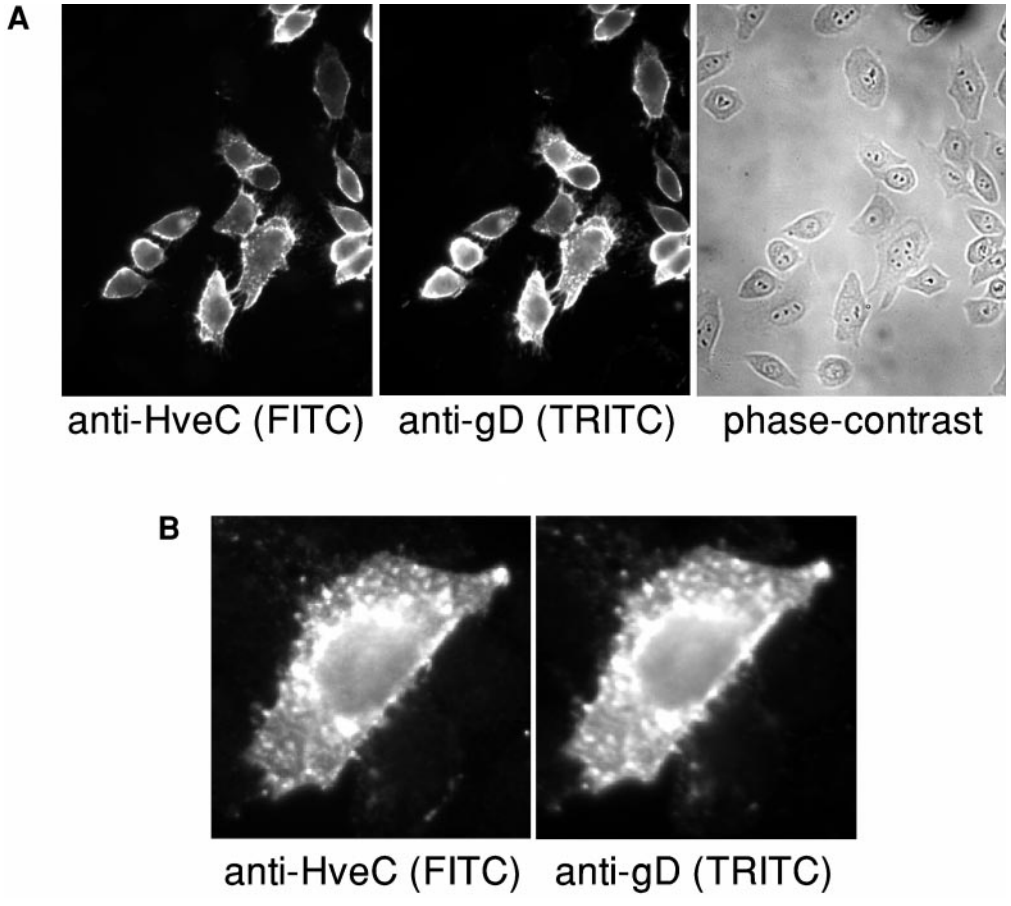

FIG. 3. Cells cotransfected with gD-expressing and HveC-expressing plasmids display both HveC and gD on cell surfaces and within the cells. CHO-K1 cells were cotransfected, at a molar ratio of 1:4, with plasmids expressing HveC (pBG38) and wild-type HSV-1 gD (pCJ3). Twenty-four hours later, the cells were replated onto coverslips. The next day, live cells were exposed to chicken anti-HveC polyclonal antibodies and rabbit anti-gD serum R7 at 4°C (A) or the cells were fixed with acetone and exposed to anti-HveC polyclonal antibodies and anti-gD serum R7 at 37°C (B). After incubation with primary antibodies, the live cells were fixed with acetone. All cells were then incubated with appropriate FITC-conjugated and TRITC-conjugated secondary antibodies, illuminated to detect the FITC or TRITC fluorescence or viewed under phase-contrast illumination using a Zeiss fluorescence Axioscope. Cells expressing either HveC alone or gD alone were also viewed using illumination to detect FITC or TRITC to ensure that the FITC signal was not detectable using TRITC illumination and vice versa.

then washed, fixed, and incubated with a detection system for quantitating the amount of rabbit Fc bound. As shown in Fig. 6, cells expressing only HveC bound gD:Fc at a much higher level than cells coexpressing HveC and each of the alphaherpesvirus gDs. Cells transfected with control plasmid alone failed to bind the gD:Fc hybrid protein. Therefore, although HveC expression was unchanged on the surfaces of cells coexpressing HveC and gD, compared to cells cotransfected with HveC and control plasmid, cell-surface HveC had significantly reduced ability to interact with exogenous gD.

Complex formation between cell-associated gD and HveC

To detect an interaction between HveC and cell-associated gD, a coimmunoprecipitation experiment was performed using lysates of cells expressing a tagged version of HveC (HveC-HA) alone, HSV-1(KOS)Rid1 gD alone, or the two proteins together. Control experiments showed that HveC-HA was indistinguishable from authentic HveC in viral entry activity and susceptibility to gD-mediated interference (data not shown). The transfected cells were lysed and immunoprecipitations were performed with the anti-HSV gD serum R7. The immunoprecipitates were electrophoresed, the proteins transferred to nitrocellulose, and the Western blots incubated either with a monoclonal antibody to HSV-1 gD, to determine the efficiency of the gD immunoprecipitation, or with an anti-HA monoclonal antibody to detect an interaction between HveC-HA and gD (Materials and Methods). The results depicted in Fig. 7 demonstrate that gD-specific antibodies coimmunoprecipitated HveC-HA from lysates of cells coexpressing HveC-HA and gD but not from lysates of cells expressing HveC-HA alone or

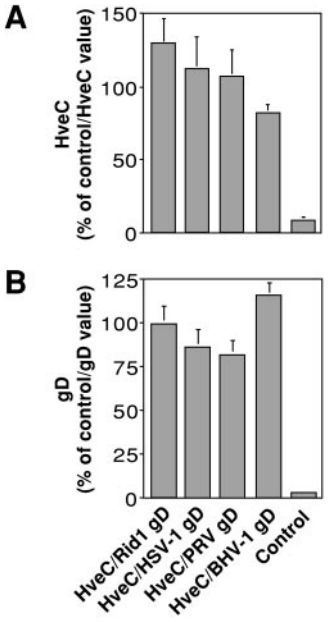

FIG. 4. Resistant cells express HveC and gD on their surfaces. Replicate cultures of the CHO-K1 cells transfected for experiments as shown in Fig. 1 were replated in 96-well plates. Twenty-four hours later, CELISA assays were performed. Live cells were exposed to primary antibodies and then washed and fixed. Binding of the primary antibodies was detected by use of biotinylated secondary antibodies and streptavidin-conjugated horseradish peroxidase. Chicken antibodies were used to detect HveC (A) and, in replicate cultures, various antibodies were used to detect the different alphaherpesvirus gDs (B). Peroxidase activity was assayed as a measure of HveC or gD antigen on the cell surface. Within each experiment, all values were expressed as a percentage of the value obtained for the HveC/control plasmid transfection (values range from 0.134 to 0.429 OD 370 nm) (A) or gD/control plasmid transfection (B) for each form of gD separately (gD/control values range between 0.5 and 0.9 OD370 nm). The experiments were performed three times and the mean values plus standard deviations for the combined results are depicted.

from control cells, indicating that complexes containing HveC and gD formed in cells coexpressing these proteins. Figure 7 also shows that coexpression of HveC with gD influences the posttranslational processing of gD. The two bands recognized by anti-gD antibodies from cells expressing gD alone have been identified as immature (lower band) and mature forms, which differ in the stage of processing of N-linked glycans from high mannose to complex and in presence of O-linked glycans (Johnson and Spear, 1983). The single gD band that predominates in lysates and immunoprecipitates from cells coexpressing HveC with gD could represent a form of gD without O-linked glycans or some other modified form of gD. Presumably the changes in posttranslational processing of gD caused by HveC coexpression are not accompanied by reduced transport of gD to the cell surface (Figs. 3 and 4). Interestingly, the HveC that coprecipitated with gD appears to be enriched for higher molecular weight forms of HveC-HA, possibly the most highly glycosylated forms.

DISCUSSION

Results presented here demonstrate that gD-mediated interference with herpesvirus infection can be explained by an interaction between cell-associated gD and available gD-binding receptors such that the receptors are not accessible to virion-associated gD even if they are present on the cell surface. We expected to find a sequestration of gD receptors inside the cell (which may occur to some extent and in some cell types) as a result of gD coexpression but found instead that the amount of HveC present on cell surfaces was not significantly reduced, if at all, when gD was also expressed. The ability of cell-surface HveC to bind to exogenous soluble gD was severely reduced, however, indicating that cell-associated gD bound to cell-surface HveC so as to occlude the binding sites for soluble exogenous gD or virion-associated gD. A physical association of HveC with gD was demonstrated by the coimmunoprecipitation of

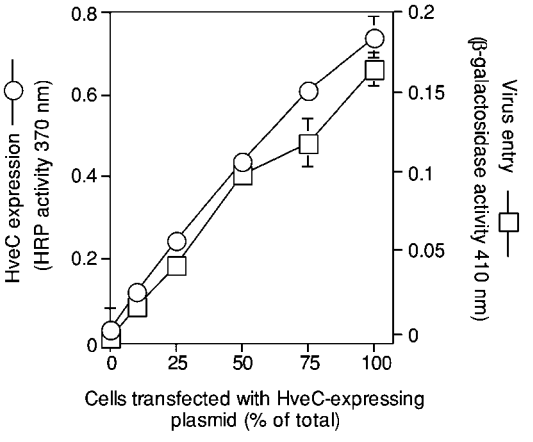

FIG. 5. Cell-surface expression of HveC and viral entry activity in mixed-cell populations. Mixed populations of cells containing an increasing percentage of HveC-expressing cells were inoculated with HSV-1(KOS)Rid1tk12 (circles) or tested for cell-surface expression of HveC (squares). CHO-K1 cells were transfected with HveC-expressing plasmid (pBG38) and control plasmid at a 1:4 molar ratio or with control plasmid (pcDNA3). Twenty-four hours later, the two transfected cell populations were mixed in various proportions and replated in 96-well dishes at 8×10^4 cells/well. To test the susceptibility to HSV-1 infection, the next day the cells were incubated with an HSV-1(KOS)Rid1 recombinant expressing β-galactosidase at a range of concentrations and β-galactosidase activity was quantitated as a measure of viral entry. The results depicted were for 5×10^5 PFU/well and in the linear range when virus dose was plotted against β-galactosidase activity. The assays were performed in triplicate and repeated two times with similar results. The mean values plus standard deviations for a representative experiment are depicted. To quantitate the levels of HveC expression, an anti-HveC CELISA was performed as for Fig. 4. Five equivalent wells per sample were analyzed in each experiment and two independent experiments were performed with similar results. The mean values plus standard deviations of a representative experiment are depicted.

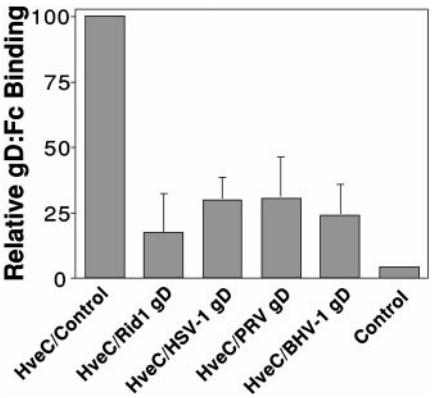

FIG. 6. HveC expressed on the surfaces of gD-expressing cells has reduced ability to bind exogenous gD. Replicate cultures of CHO-K1 cells transfected for experiments as shown in Fig. 1 were replated in 96-well plates. The next day the cells were exposed to gD:Fc, washed, fixed with formaldehyde and glutaraldehyde, and incubated with biotinylated secondary antibodies followed by streptavidin-conjugated horseradish peroxidase. Peroxidase activity was assayed as a measure of gD:Fc binding to the cell surface. Within each experiment, all values were made relative to the value obtained for the HveC/control plasmid cotransfection (HveC/control values ranged between 0.09 and 0.4 OD 370 nm). Cells transfected to express each of the gDs alone were included in these experiments. No significant binding of gD:Fc to cells expressing only gD was detected (data not shown). The experiments were performed three times and the mean values plus standard deviations for the combined results are depicted.

HveC with gD. One implication of these findings is that HveC–gD interactions required for interference may be similar or identical to those required for viral entry. Thus, an alphaherpesvirus that cannot use one of the gD receptors for entry, due to failure to bind the receptor, should encode a form of gD that has little or no ability to interfere with use of this receptor by other viruses. Consistent with this prediction, wild-type HSV-1(KOS) gD interferes with entry of viruses via HveA (Mauri *et al.*, 1998) or HveC but not HveB, whereas mutant HSV-1 (KOS)Rid1 gD can interfere with entry via HveB (Fig. 2).

The basis for cross-interference among alphaherpesviruses can be the ability of these viruses to use the same gD receptor for entry. Each form of alphaherpesvirus gD tested was able to interfere with entry of the homologous and heterologous viruses, presumably because the only gD receptor available was HveC, which can mediate entry of all the viruses used. In previous studies nonreciprocal cross-interference was observed. For example, bovine cells expressing BHV-1 gD were resistant to entry of both BHV-1 and PRV, whereas the same cells expressing PRV gD were resistant to PRV entry but not BHV-1 entry (Chase *et al.*, 1990, 1993). A possible explanation is that multiple gD receptors were expressed in the bovine cells, some of which mediate entry of both BHV-1 and PRV and at least one of which mediates entry of BHV-1 only.

The fact that interference was observed with all combinations of gD and challenge virus tested indicates that each form of alphaherpesvirus gD interacts with overlapping domains of HveC. We suggest that the relative degree of interference observed with each form of gD reflects the relative affinity of its interaction with HveC. This proposed correlation is supported by findings that a soluble form of HSV-1(KOS)Rid1 gD binds to soluble HveC with higher affinity than does a similar form of wild-type HSV-1(KOS) gD (Krummenacher *et al.*, 1999). In addition, the different forms of gD could be ranked according to their interference activity and this ranking was the same regardless of challenge virus (Fig. 1). Finally, the virus encoding gD with least interference activity (BHV-1) was also most sensitive to interference regardless of the form of gD expressed, indicating that the BHV-1 form of gD competed poorly with all forms of cell-associated gD for receptor.

Interestingly, HSV-1 and PRV gDs had nearly equivalent interference activities despite only 27% sequence identity. On the other hand, the wild-type and Rid1 forms of HSV-1 gD differ by only one amino acid and yet differ in interference activity. The fact that several divergent and similar forms of gD can apparently compete for binding sites on human HveC should facilitate identification of the structural features of gD required for the binding.

Interference assays provide a powerful tool for characterizing the interaction between viral glycoproteins and entry receptors and could be used to identify the

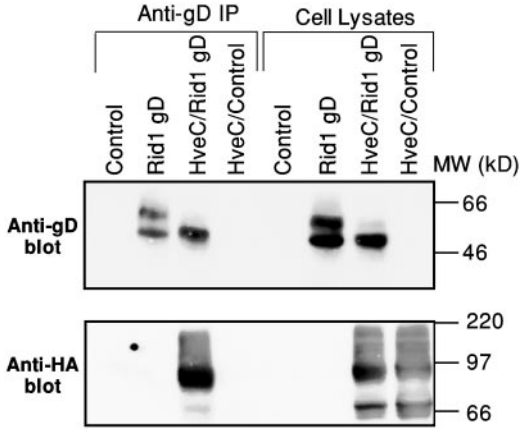

FIG. 7. Coexpression of HveC and HSV-1 gD results in an HveC-gD interaction. PEAK cells (Edge BioSystems) were transfected, at a molar ratio of 1:1, with plasmids expressing HveC-HA (pCJ4) and Rid1 gD (pCJ1), HveC-HA-expressing plasmid (pCJ4) and control plasmid (pcDNA3), Rid1 gD-expressing plasmid (pCJ1) and control plasmid (pcDNA3), or control plasmid (pcDNA3) alone. Forty-eight hours later, the cells were detached, lysed for immunoprecipitation with rabbit polyclonal anti-HSV gD serum R7, or lysed with sample buffer and analyzed by SDS–PAGE. After electrophoresis, the separated proteins were transferred to nitrocellulose and Western blot analysis was performed to detect gD (anti-gD monoclonal antibody DL6) or HveC-HA (rat anti-HA monoclonal antibody) in the gD immunoprecipitates or in the cell lysates. After incubation with the primary antibodies, the membranes were incubated with appropriately conjugated secondary antibody and visualized via enhanced chemiluminescence.

viral ligands for entry receptors in cases in which the virus expresses multiple candidates for such ligands. Interference assays can also be used to determine whether different viruses can use the same receptor for entry, as has been well-documented for retroviruses (Kewalramani *et al.*, 1992; Miller, 1996; Sommerfelt and Weiss, 1990). Finally, interference assays provide a facile means of characterizing functional domains of the interfering viral protein. Mutations in gD that abrogate interference activity (without global effects on overall protein structure) are likely also to eliminate entry activity, assuming that the gD–HveC interactions required for both activities are sufficiently similar.

What is the physiological relevance of envelope glycoprotein-mediated interference with viral entry? In the case of retroviruses, expression of envelope glycoprotein in infected cells prevents superinfection with homologous or related viruses. Since pathogenesis or spread of these viruses often depends on continued survival and replication of productively infected cells, superinfection exclusion can prevent multiple provirus insertions and promote cell survival. Retrovirus interference is often associated with reduction in levels of cell-surface receptors, which could aid in release of virus from infected cells and might also alter normal cell functions dependent on cell-surface expression and accessibility of the receptors. In the case of alphaherpesviruses, gD is expressed only in lytically infected cells and it is not clear whether superinfection of lytically infected cells occurs or is of any consequence. It has been postulated that gD-mediated interference is important for efficient egress and release of infectious virus by preventing newly enveloped virus from fusing with membranes of the virus-producing cell (Campadelli-Fiume *et al.*, 1990; Johnson and Spear, 1989). It is also possible that interaction of gD with its various receptors could alter the activities of these receptors. Under certain conditions binding of gD to HveA can prevent binding of HveA to its natural ligands, members of the tumor necrosis factor family (Mauri *et al.*, 1998). Recently, HveB and HveC have been shown to function as homophilic adhesion molecules that localize to intercellular junctions (Takahashi *et al.*, 1999). The expression of gD within epithelial cells at the initial site of infection could inhibit the localization of HveC to these junctions and perhaps affect the junctional integrity. This disruption may aid in the spread of infectious virus and possibly even facilitate spread of virus from the initial site of infection to neurons for the establishment of latency.

MATERIALS AND METHODS

Cells and viruses

CHO-K1 cells were provided by J. Esko (University of California at San Diego) and were grown as previously described (Montgomery *et al.*, 1996). The PEAK cells (Edge Biosystems) were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics. The β-galactosidase-reporter viruses used have been described previously, HSV-1(KOS)tk12 and HSV-1(KOS)Rid1tk12 (Warner *et al.*, 1998), PRVgH$^-$ (Babic *et al.*, 1996) provided by T. Mettenleiter (Federal Research Centre for Virus Diseases of Animals, Insel Riems, Germany), and BHV-1(Cooper)v4a (Miller *et al.*, 1995) provided by L. Bello (University of Pennsylvania). The HSV strains were propagated on HEp-2 cells and titered on Vero cells, PRVgH$^-$ was propagated and titered on gH-expressing VeroSW78 cells, and BHV-1(Cooper)v4a was propagated and titered on MDBK cells. Sindbis virus (strain TRSB) expressing green fluorescent protein (Klimstra *et al.*, 1998) was provided by W. Klimstra and R. Johnston (University of North Carolina at Chapel Hill).

Plasmids

All expression plasmids use the human cytomegalovirus (CMV) immediate-early promoter for efficient protein expression in eukaryotic cells. The construction of the HveC- and HveB-expression plasmids, pBG38 and pMW20, respectively, has been described (Geraghty *et al.*, 1998; Warner *et al.*, 1998). A plasmid expressing a tagged version of HveC (pCJ4) was constructed by amplifying the HveC open reading frame (ORF) with the primers cD3prim (5′CACTGCTTACTGGCTTATCG) and BamHveC (5′GCGGATCCGCCACGTACCACTCCTTCTTGG), digesting the amplified product with *Bam*HI, and inserting that fragment into pMN104 (Montgomery *et al.*, 1996) via the *Bam*HI site. The protein expressed by pCJ4 is designated HveC-HA and consists of the entire HveC ORF fused at its carboxy-terminus to the influenza A hemagglutinin (HA) epitope YPYDVPDYA (Wilson *et al.*, 1984). The plasmids expressing the HSV-1(KOS)Rid1 or wild-type HSV-1 form of gD, pCJ1 or pCJ3, were constructed by excising the gD gene from plasmids pMW13 or pRE4 (Cohen *et al.*, 1988), respectively, via flanking *Hin*dIII restriction sites, and inserting the genes into the *Hin*dIII site of pcDNA3 (Invitrogen). The plasmid MW13 was provided by M. Warner and constructed by replacing the gD gene in pRE4 with the Rid1 form of gD taken from pHD32 (Dean *et al.*, 1994) using the *Afl*III and *Acc*I restriction enzyme sites. The PRV gD-expressing plasmid pCMVgD, provided by V. Gerdts (Federal Research Centre for Virus Diseases of Animals), has been described (Gerdts *et al.*, 1997). The BHV-1 gD-expression plasmid, pBG58, was constructed by digesting pMAGD (Fehler *et al.*, 1992), provided by G. Keil (Federal Research Centre for Virus Diseases of Animals), with *Hin*dIII and *Avr*II to isolate a fragment containing the gD open reading frame and inserting the fragment into the *Hin*dIII and *Xba*I sites in pcDNA3. The plasmid expressing the gD:Fc fusion protein, pBG64, was constructed by isolating the *Hin*dIII to *Xba*I fragment containing the gD:Fc gene from pBL50

and inserting it into the *Hin*dIII and *Xba*I sites in pcDNA3. The plasmid BL50, provided by B. Lum, contains an ORF encoding the entire extracellular domain of HSV-1(KOS) gD up to the *Sgr*AI site at the beginning of the transmembrane domain fused to the hinge, CH_2, and CH_3 regions of the rabbit IgG heavy chain. To facilitate construction, a linker region was placed in frame between the gD domain and the Fc domain beginning with the 5′ CG from the *Sgr*AI site in gD: 5′CGCCGGGTACCGAGCTCGAATTCACAAGACCGTTG (the underlined nucleotides represent the 5′ sequences of the Fc region). The Fc region of the fusion protein was derived from Clone 3-4 provided by K. Knight (Loyola University Medical Center).

Antibodies and hybrid protein

The chicken polyclonal anti-HveC antibodies were generated at Aves Laboratories by inoculation of laying chickens with the extracellular domain of HveC (HveC346t), provided by G. Cohen and R. Eisenberg (Krummenacher *et al.*, 1998), and total IgY was purified from the eggs. The rabbit polyclonal anti-HveB serum R146 (Warner *et al.*, 1998), the rabbit anti-HSV gD serum R7 (Isola *et al.*, 1989), the anti-gD monoclonal antibody DL-6 (Cohen *et al.*, 1986), the anti-PRV gD monoclonal antibody 6D8MB4 (ATCC), and the anti-BHV-1 gD monoclonal antibody 1B8-F11 (ATCC) were provided by G. Cohen and R. Eisenberg. The rat anti-HA high-affinity monoclonal antibody was purchased from Boehringer Mannheim (Cat. No. 1867423). To produce the gD:Fc protein, PEAK cells were transfected with pBG64 by the calcium phosphate method (according to the Edge Biosystems' protocol), and the secreted protein was purified using protein A/G columns (Pierce) following the manufacturer's instructions. The purified gD:Fc protein was shown to bind specifically to cells transfected to express HSV-1 gD receptors, including HveA (unpublished), HveC (this study—Fig. 6), and 3-OST-3-modified heparan sulfate (Shukla *et al.*, 1999b), but not to cells devoid of such receptors. A full description of the properties of this hybrid protein will be published elsewhere (A. Fridberg, R. J. Geraghty, B. Lum, and P. G. Spear, manuscript in preparation).

Interference assay

Subconfluent CHO-K1 cells were transfected with 5 μl LipofectAMINE reagent and 1.5 μg of plasmid DNA per well of a 6-well dish according to the manufacturer's instructions. The molar ratio of gD plasmid or control plasmid (pcDNA3) to HveC- or HveB-expressing plasmid was 4:1. Twenty-four hours later, the cells were replated into 96-well dishes (approximately 2×10^4 cells per well). Twelve to 24 h later, the cells were inoculated with virus diluted in phosphate-buffered saline (PBS). After 1.5 h, the virus inoculum was removed, and the cells were treated with 0.1 M citrate buffer (pH 3) and washed three times with cell culture medium. Approximately 4.5 h later, the cells were lysed and β-galactosidase activity was determined to measure virus entry as described (Montgomery *et al.*, 1996).

Sindbis virus–GFP infections were performed as described (Klimstra *et al.*, 1998). Briefly, cells transfected as above and replated into 24-well dishes were inoculated for 60 min at 37°C with 3×10^7 PFU in PBS. The monolayers were washed three times with PBS and incubated in growth medium for 7 h. The cells were removed from plates using PBS/4 mM EDTA, washed with ice-cold growth medium, resuspended in cold PBS/2% heat-inactivated-calf serum, and analyzed by flow cytometry to determine the percentage of infected cells.

CELISA assay and gD:Fc binding assays

CHO-K1 cells were transfected as described above and, after 24 h, were plated into 96-well dishes (approximately 4×10^4 cells per well). The next day, the cells were incubated with primary antibodies in 50 μl PBS/3% BSA for 30 min at room temperature at the following dilutions: polyclonal chicken anti-HveC at 1 μg/ml, polyclonal rabbit anti-HveB serum R146 at 1:5000, polyclonal rabbit anti-HSV gD serum R7 at 1:20,000, monoclonal anti-PRV gD 6D8MB4 ascites fluid at 1:5000, monoclonal anti-BHV gD 1B8-F11 ascites fluid at 1:1000, and the gD:Fc fusion protein at 1 μg/ml. (The anti-HveC and anti-HveB sera were preadsorbed against CHO-K1 cells at room temperature for 15 min before use.) After primary incubation, the cells were washed five times with PBS and fixed with 100 μl 2% formaldehyde and 0.2% glutaraldehyde for 10 min at room temperature. The cells were washed three times with PBS/3% BSA and incubated with biotinylated secondary antibodies against rabbit IgG (Sigma), mouse IgG (Sigma), or chicken IgY (Vector Laboratories) at a 1:500 dilution in 100 μl PBS/3% BSA for 30 min at room temperature. Following the secondary incubation, the cells were washed five times with PBS and incubated with AMDEX streptavidin-conjugated horseradish peroxidase (Amersham) at 1:20,000 dilution in 100 μl PBS/3% BSA/0.1% Tween 20 for 30 min at room temperature. Following tertiary incubation, the cells were washed five times in PBS/0.1% Tween 20 and incubated with 3,3′,5,5′-tetramethylbenzidine in phosphate-citrate buffer as per the manufacturer's instructions (Sigma). At various times after addition of substrate, the plates were read at 370 nm in a Spectra Max 250 ELISA reader.

Immunoprecipitation and Western blot analyses

PEAK cells were transfected with plasmids expressing HveC-HA (pCJ4) or HSV-1(KOS)Rid1 gD (pCJ1) mixed 1:1 with pcDNA3 or with a mixture of pCJ4 and pCJ1 using the calcium phosphate transfection method (Edge Biosystems). For these experiments, a 1:1 ratio of gD:HveC-

HA plasmids was used to facilitate detection of HveC-HA. Forty-eight hours after transfection the cells were harvested and lysed on ice for 10 min in 1 ml of ice-cold lysis buffer containing 0.5% Triton X-100, 10% glycerol, 10 mM Tris–HCl, pH 7.5, 145 mM NaCl, 5 mM EDTA, and aprotinin, leupeptin, and pepstatin each at 10 μg/ml. The cell lysates were clarified by centrifugation for 15 min at 4°C. The lysates were then precleared with 75 μl of protein A/G (Pierce) for 1 h at 4°C. At the same time, 75 μl of protein A/G was added to 15 μl of rabbit anti-HSV gD serum R7, in 1 ml of cold PBS, and incubated at 4°C for 1 h. The precleared lysate was incubated with the R7-protein A/G for 1 h at 4°C. The immune complexes were collected by centrifugation at 11,000 rpm in a microcentrifuge for 2 min at 4°C. The complexes were washed three times with lysis buffer over 30 min, boiled for 4 min in SDS sample buffer containing 100 mM DTT, and separated on a 10% SDS–polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature in PBS containing 0.1% Tween 20, 5% milk powder, and 1% BSA. Immunoblots were performed with a 1:5000 dilution of anti-HA monoclonal antibody or a 1:5000 dilution of gD monoclonal antibody DL6 in blocking buffer for 1 h at room temperature. The blots were washed three times for 10 min in PBS containing 0.1% Tween 20. Horseradish peroxidase-conjugated anti-rat or anti-mouse secondary antibodies (Boehringer Mannheim) were incubated with the blots for 30 min (1:5000 dilution in blocking buffer). The blots were then washed three times for 10 min with PBS containing 0.1% Tween 20. The proteins were visualized by enhanced chemiluminescence system following the manufacturer's protocol (Amersham Pharmacia Biotech).

Immunofluorescence

CHO-K1 cells were transfected as described above and 24 h later replated onto glass coverslips. Twenty-four hours later, the cells were stained. For cell-surface immunofluorescence, the cells were incubated with both primary antibodies at 4°C for 45 min, washed 10 times with cold PBS, and fixed with acetone for 10 min at −20°C. The polyclonal chicken anti-HveC antibodies were diluted 1:200 in PBS/10% normal goat serum (NGS) and the anti-gD polyclonal rabbit serum was diluted 1:500 in PBS/10% NGS. After fixation, cells were dried at room temperature for 5 min, blocked in PBS/10% calf serum, and incubated with FITC-conjugated and TRITC-conjugated secondary antibodies prepared in goats against chicken IgY (Aves Laboratories) and rabbit IgG (Jackson Laboratories), respectively, at a dilution of 1:80 in PBS/10% NGS, for 30 min at 37°C. Following secondary antibody incubation, the cells were again washed 10 times in PBS. For immunofluorescence on permeabilized cells, the cells were fixed with acetone for 10 min at −20°C, dried at room temperature, blocked in PBS/10% calf serum for 30 min, and incubated with primary antibodies for 30 min at 37°C. The polyclonal anti-HveC antibodies were diluted 1:500 and the polyclonal anti-gD serum R7 was diluted 1:1000 in PBS/NGS. After incubation with primary antibodies, the cells were washed 10 times with PBS and incubated with secondary antibodies as described above. All coverslips were mounted on slides using mounting solution (40 mM Tris, pH 8.0, 75% glycerol, 0.1% *p*-phenylenediamine) and viewed using a Zeiss fluorescence Axioscope.

Flow cytometry

CHO-K1 cells were transfected as described above and after 48 h detached with PBS/4 mM EDTA. Approximately 5×10^5 cells were washed with 1 ml ice-cold PBS/2% calf serum and incubated with primary antibodies on ice in 100 μl for 10 min. The anti-gD serum R7 was diluted 1:500 in PBS/2% calf serum and the chicken polyclonal anti-HveC was used at 100 μg/ml. The cells were washed and incubated in PBS/2% calf serum plus secondary antibody for 10 min in 100 μl on ice. FITC-conjugated goat anti-rabbit (Sigma) and biotinylated donkey anti-chicken (Jackson ImmunoResearch) were diluted 1:200. The cells were washed and incubated in ice-cold PBS/2% calf serum containing APC-conjugated streptavidin (Pharmingen) at a dilution of 1:200 for 10 min. Just prior to performance of flow cytometry (Becton–Dickinson FACS-Calibur) propidium iodide was added to a final concentration of 1 μg/ml in PBS/2% calf serum to exclude dead cells from analysis.

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